Cytological Examination of *Scilla peruviana* L. during a 20-month Cycle of Growth and Development

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Abstract. The growth cycle of *Scilla peruviana* L. involved the development of two generations of daughter bulbs enclosed within each mother bulb. Flower initiation of the primary daughter bulb took place in June as the mother bulb apparently entered dormancy. Floral differentiation was complete by late October, by which time the apical meristem of the secondary daughter bulb had developed for 3 months inside the primary daughter bulb. The complete cycle of ontogenesis, from meristem initiation to flowering, occurred without interruption and required 20 months. Small zones of meristematic cells detected at the bases of bulb scales may be the origin of adventitious bulblets in this species. This detailed cytological study enabled the development of an effective commercial forcing program for *S. peruviana*.

Scillas are bulbous plants whose distribution extends from South Africa, north to the Mediterranean and into Europe and Asia. *Scilla* belongs to the subclass Monocotyledonae, and the family Liliaceae (Bryan, 1989; Van Sheepen, 1991) or Hyacinthaceae (Du Plessis and Duncan, 1989). *Scilla* species have great potential as valuable potted and garden plants (Le Nard and De Hertogh, 1993) but, while their ornamental qualities have been recognized for centuries, critical examination of these flowering bulbs has been minimal. The genus includes >100 species, of which only a few are grown commercially in the Netherlands and the United States (Langeslag, 1989). Commercial production of *Scilla* has focused on *Scilla tubergeniana* L., and information on growth, development, and flowering of this species is summarized by Le Nard and De Hertogh (1993).

One of the most attractive *Scilla* species is *S. peruviana*. Its storage organ is a tunicated bulb that is renewed annually. *S. peruviana* is propagated by seeds or vegetatively, but since few bulblets are produced, the multiplication rate is very low (Langeslag, 1989). Information is not available on the control of floral development in this species.

Neither regulation of flowering time nor increasing the multiplication rate can be achieved without knowledge of the basic periods of ontogenesis of the plant. Defining the processes of initiation and differentiation of the apical meristem of the bud is especially important. Within this apical shoot meristem, morphogenetic changes result in the formation of various organs during specific periods of growth and development of the bulbs. Determining the precise timing of the development of the apical shoot meristem from initiation to complete differentiation of flower organs enables growers to control bulb growth and flowering. The correlation between the physiological state of the bulbs and cytological changes occurring in the apical shoot meristems also makes possible the regulation of growth, development, and multiplication, both in vivo and in vitro, by modulating external factors such as temperature, humidity, and growth regulator application.

The present study focused on two aspects of *S. peruviana* bulb development. First, we studied the morphological structure of *S. peruviana* bulbs during a complete cycle of ontogenesis, from flowering to flowering. Second, special attention was given to cytological changes at the apical shoot meristem from bud initiation until differentiation of flower organs. Because bulbs are commonly propagated in vitro and in vivo using twin scales, we also investigated the cytophysiological changes that occur in scales during their growth and development.

Materials and Methods

Scilla peruviana bulbs were obtained from Hanbury Botanical Garden in Ventimiglia, Italy, and used in experiments from Oct. 1994 to July 1996 in the university greenhouses (lat. 49°18'56"N, long. 123°04'44"W). At the end

of September, bulbs (14- to 15-cm circumference, ≈175 g) were planted in 15-cm pots in a peat-based potting medium (Metro-Mix 290; Grace Horticulture Products, Ajax, Ont.). Plants were grown in a greenhouse under natural photoperiods with 13 °C day/10 °C night temperatures. A 20N–8.7P–16.6K water-soluble fertilizer (W.R. Grace & Co., Fogelsville, Pa.) was applied as a drench at the rate of 2.4 g·L⁻¹ during a low-frequency irrigation program. After flowering and leaf senescence, bulbs were lifted (10–15 June, over 2 years), cleaned and dipped in benomyl [methyl 1-(butylcarbamoyl)-2 benzimidazolecarbamate] fungicide (Dupont Canada Inc., Mississauga, Ont.; 0.2 g·L⁻¹ for 1 h). The bulbs were then dried and stored at 25 °C for 10 d, followed by storage at 17 to 19 °C until potting in early October.

In both years, once every month, three to five bulbs were examined. On each sampling date, bulbs were dissected to record their stages of development, including bulb diameter, size of buds, length of flower stem (including inflorescence), inflorescence height, and number of leaves. Observations are reported for 2-month intervals, focusing on the major differences observable throughout ontogenesis.

For cytological examination, buds and twin scales from each bulb were isolated. These were fixed in Carnoy's solution (6 ethanol : 3 chloroform : 1 acetic acid; by volume) for 24 h, or FAA [5 formalin : 5 acetic acid : 9 (50%) ethanol; by volume] for 12 h, dehydrated in a graded series of ethanol/xylene, and embedded in a paraffin wax mixture according to Sass (1958) with the following modifications. Samples were transferred from Carnoy's solution (or FAA) to a series of ethanol concentrations, including once each in 70% and 95% and 3× in absolute ethanol, each for 2 h. They were then transferred to a series of 30%, 50%, 75%, and 95% xylene in absolute ethanol and 4× in 100% xylene, each for 24 h. Tissues were cut longitudinally (7-μm sections) on a rotary microtome (Reichert, Austria). Sections were stained with Delafield's hematoxylin and eosin (Sass, 1958), and examined by light microscopy, and micrographs were prepared.

Mitotic index (MI), the percentage of mitotic cells in a defined area, was studied in two experiments by counting the number of nuclei and the number of mitoses (excluding prophase) in a defined area. Samples were obtained from six serial sections, each from three-bulb replicates. In one experiment (Mar. 1995–Apr. 1996), MI of the undifferentiated apical meristem area was determined. Since the sizes of the apical meristem areas changed throughout ontogenesis, the mean number of cells counted (±SE, n = 3) is reported for each mean MI (±SE, n = 3). In another experiment (July–Dec. 1995), the MI of apical meristems undergoing floral differentiation was determined. Samples were obtained from the most apical and most basal florets of a single inflorescence. Data were analyzed as a split-plot design with months as the main plot and apical vs. basal floret as the subplot, using SAS (SAS Institute, 1996).

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Fig. 1. Annual growth and development of *Scilla peruviana*. Pictures within each column represent the same time of year. (A-F) Growth of whole plant. (a) Thick fleshy scales (leaf bases from previous years); (b) thin fleshy scales (leaf bases of the current year); (c) outer papery scales (tunics); (d) basal plate with roots; (e) leaves; (f) inflorescence; (g) primary daughter bulb; (h) secondary daughter bulb. (G-L) Longitudinal sections through the apical meristem of the primary daughter bulb during ontogenesis. Apical meristem (Am), leaf primordium (L), floral primordium (Fp), bract (B), petal (P), stamen (St), gynoecium (G), pollen mother cells (Pmc), pollen grains at the tetrad stage (Pg), ovule (O). (M-P) Longitudinal sections through the apical meristem of the secondary daughter bulb during ontogenesis. Flower stem (Fs). (Q-V) Longitudinal sections through the basal plate and scales of the mother bulb. Meristematic cells (Mc), bulb scale (Sc). Scale bar = 0.1 mm. (Fig. 1 continued on next page.)

Fig. 1 continued.

Results

The egg-shaped bulb of *S. peruviana* is tunicated and composed of outer and inner scales (Fig. 1A.a-c). The outer scales (enlarged leaf bases of the previous years) are white, fleshy, and swollen with nutrients and moisture, except for the outermost, dry, papery scales (tunics). The inner scales (bases of this

year's leaves) are more leaflike and protect the central growing shoot. All scales are attached to a basal plate from which adventitious roots arise (Fig. 1A.d). The growth and development of *S. peruviana* during ontogenesis is summarized in Fig. 1.

March-April. In our greenhouses, *S. peruviana* flowered from late March to the end of April. At this time, the strap-shaped leaves

formed a rosette at the base of the flower stem. The inflorescence was composed of up to 100 lilac-blue, star-like blooms that opened successively from the base to the apex of the inflorescence (Fig. 1A.f). Alongside the flower stem was a bud or primary daughter bulb, which is destined to flower the following year (Fig. 1A.g). The height of the primary daughter bulb when the mother bulb was flowering

ranged from 0.3 to 0.5 cm. The apical meristem of the primary daughter bulb was a hemispherical dome $\approx 380 \mu\text{m}$ in diameter (Fig. 1G) with a mitotic index (MI) of 2.4 ± 0.2 (1296 ± 1 cells counted). On the perimeter of the apical dome, the initiation of five to six leaf primordia was observed (Fig. 1G). Cytological examination of a longitudinal section of the bases of the scales and basal plate revealed a distinct group of meristematic cells that could be distinguished readily from the surrounding cells (Fig. 1Q). These meristematic cells had large nuclear volumes and their cytoplasm was vacuolated.

May–June. The leaves and roots of the mother bulb began to dry up gradually from May–June (Fig. 1B). At this stage, the growth of the primary daughter bulb accelerated; it ranged in height from 0.6–0.9 cm (Fig. 1B.g). Significant changes were discernible in longitudinal sections of the apical meristem during this period of ontogenesis. The apex increased in size ($608\text{-}\mu\text{m}$ mean diameter) and became domed (Fig. 1H). Cells of the apical meristem became enlarged and vacuolated. The structures of cells and nuclei changed rapidly in June, accompanied by increased mitotic activity ($\text{MI} = 5.7 \pm 0.3$; 7563 ± 495 cells counted). The first flower primordium appeared during this period. These changes indicated the transition of the apical meristem of the primary daughter bulb from vegetative to prefloral development. Eight to 10 leaf primordia were also initiated by the apical meristem. An increased number of meristematic cells was observed between scales during this period of ontogenesis (Fig. 1R).

July–August. The concealed, underground period in the development of *S. peruviana* bulbs occurred from July through August. During the “dormancy” period of the mother bulb, the primary daughter bulb continued to grow. Sampled bulb height ranged from 3.5 to 5.0 cm (Fig. 1C.g). The apex of the inflorescence was also much larger (0.4-cm diameter) than in the previous stage (Fig. 1C.g). Intensive, successive processes of differentiation of bract, petal, and stamen primordia were observed (Fig. 1I). This reorganization of the apical meristem was associated with a high level of mitotic activity (described in detail below) and with pronounced cell vacuolation and elongation. Foliar initiation ceased in this period after generating a total of 13 to 15 leaves. The initiation of the apical meristem of the secondary daughter bulb was observed at the base of the flower stem of the primary daughter bulb, which was itself enclosed within the mother bulb (Fig. 1M).

The new apical meristem was slightly convex and averaged $150 \mu\text{m}$ in diameter. Cells and nuclei were small, with an MI of 1.4 ± 0.2 (901 ± 39 cells counted). In May–June, before initiation of the secondary daughter bulb, the MI at the site of initiation between the flower stem and bulb scale was 0.65 ± 0.08 (1827 ± 111.5 cells counted). Leaf primordia were not yet defined on the secondary daughter bulb in July–August. The zone of meristematic cells detected between scales disappeared in this period (Fig. 1S).

September–October. During these months, new leaves and roots emerged from the bulb. In the primary daughter bulb, floral organogenesis in the lower part of the inflorescence (0.8 cm-inflorescence height) appeared to be complete, with the formation of stamens and gynoecium (Fig. 1P). The apical meristem of the secondary daughter bulb changed its configuration slightly, showing a convex apex ($234\text{-}\mu\text{m}$ mean diameter) covered by a leaf primordium. The dimensions of the cells and nuclei remained virtually unchanged and the MI remained low at 1.6 ± 0.1 (1036 ± 26 cells counted). A small group of meristematic cells reappeared between the scales (Fig. 1T).

November–February. Intensive growth occurred during the winter months as the leaves, roots, and flower stem attained their maximal development (Fig. 1E and F). The flower stems of the primary daughter bulbs reached heights of 3.5 to 6.0 cm and the inflorescences became much larger (1.6 cm in height) and more mature. The anthers contained microspores in the premeiotic pollen mother cell stage, while the gynoecium had differentiated into ovary and style (Fig. 1K). The apical meristem of the secondary daughter bulb continued to develop (Fig. 1O), with the apical diameter averaging $343 \mu\text{m}$ and having a more pronounced convex form. The apical MI was 2.0 ± 0.1 (1638 ± 52 cells counted). Three to four leaf primordia could be distinguished.

The flower head emerged from the bulb at the end of February. Cytological examination revealed enlargement and maturation of the inflorescence, which contained tetrad-stage pollen grains and young ovules (Fig. 1L). The dome-shaped apical meristem of the secondary daughter bulb increased further in size

($360\text{-}\mu\text{m}$ mean diameter) and had initiated five to six leaf primordia (Fig. 1P). The MI was 2.0 ± 0.0 (1997 ± 79 cells counted). At this time, the number of meristematic cells observed between the scales increased (Fig. 1V).

Flowering of the primary daughter bulb in March–April of the 2nd year completed the cycle of development. The secondary daughter bulb initiated in July–August of the previous year was now designated the primary daughter bulb. Its MI at this time was 2.5 ± 0.1 (1520 ± 28 cells counted), which is comparable to the MI of the primary daughter bulb in March–April of the previous year.

Mitotic activity during flower development.

During early floral organogenesis (July–August), florets were observed at different stages of development within an inflorescence (Fig. 1I). Florets at the base of the inflorescence were more developed than those closer to the apex. This corresponded to an increase in mitotic activity that also progressed from the base to the apex of the inflorescence (Fig. 2). Inflorescences sampled in November–December no longer exhibited such a contrast, apparently coinciding with the full development of the inflorescence. To examine this more closely, we determined the MI of the most apical and most basal florets within an inflorescence during the period of floral development from July through December. Differences in MI between the most apical and most basal florets changed over time ($P = 0.0001$) (Fig. 3).

Discussion

Investigation of the annual growth and development of *S. peruviana* over a 2-year period permitted us to characterize its com-

Fig. 2. Variation in mitotic index (MI; percentage of cells in mitosis) with floret location. The MI of each floret is shown with respect to floret location in a longitudinal section of a typical inflorescence (drawn at right; not to scale). The number of florets differs in each graph. A. July–August; B. September–October; C. November–December.

Fig. 3. Mitotic index (percentage of cells in mitosis) of apical (●) and basal (■) florets of an inflorescence during floral development. Each point is the mean $\pm$ SD of three single-bulb replicates. A lack of error bar indicates a SD smaller than the symbol size. Significance of effects: months, $P \leq 0.01$; apical vs. basal floret, $P \leq 0.001$; months $\times$ apical vs. basal floret, $P \leq 0.0001$ by ANOVA.

plete cycle of ontogenesis from initiation of the apical meristem in July–August until flowering 20 months later in March–April. While the absolute timing of development will vary with environmental conditions, the physical features of the bulb described here (bulb diameter, flower stem height, inflorescence height, and leaf and root growth and senescence) can be used as reliable indicators of the developmental stages of the meristems, which we have characterized cytologically.

At the time of flowering we identified a small bud, or primary daughter bulb, at the stage of leaf initiation. Many workers (Bernier, 1971; Nougarede, 1985) have shown that transition of the apical meristem from the vegetative to reproductive stage is accompanied by specific structural and metabolic changes, including an increase in sizes of cells and nuclei, a sharp rise in the rate of cell division (mitotic activity), vacuolation, and reorganization of the structure of the apex. Similar changes were found in the apical meristem of the primary daughter bulb in June. Inflorescence initiation occurred 9 to 10 months before anthesis, simultaneous with the senescence of leaves and roots of the mother bulb in June.

In July–August, the mother bulb of *S. peruviana* is in an apparent state of dormancy. However, our observations of cytological changes and high mitotic activity in the apical meristem showed that active flower differentiation in the primary daughter bulb is under way during this time. In addition, a new vegetative apex (secondary daughter bulb) is initiated within the primary daughter bulb, which is itself enclosed within the mother bulb.

New leaves and roots of the mother bulb start to appear in September–October, coinciding with flower differentiation in the primary daughter bulb and leaf initiation in the secondary daughter bulb. *S. peruviana* bulbs are usually planted at this stage. Note that the

florets within the primary daughter bulb inflorescence were in different stages of gynoecium development. From an operational perspective, during forcing, exposure of these bulbs to very low temperature (e.g., 3 to 5 °C) could result in abortion of immature upper florets of the inflorescence. Their high MI suggests that they are still undergoing intensive nuclear division, and thus have not completed floral organogenesis in September–October.

Intensive leaf and root growth of the mother bulb observed from November to February coincided with maturation and elongation of the flower stem of the primary daughter bulb, and continuation of leaf initiation in the secondary daughter bulb. The low MI of florets throughout the inflorescence indicates that they should not be harmed by cold treatment at this stage.

Gilford and Rees (1974) reported that the life span of a tulip from bud initiation to its senescence takes 41 months, and passes through three periods of inactivity of the apical meristem. The life cycle of *Ornithogalum arabicum* L. from the formation of a vegetative bud to flowering takes ≈ 20 months. During this time, the vegetative apex is inactive for 4 months (Shoub and Halevy, 1971). In *S. peruviana*, however, the development of the apical meristem from initiation until flowering takes 20 months, and occurs without interruption. The apical meristem produced leaves for ≈ 11 to 12 months in the first year, followed by differentiation and maturation of the flower organs over the subsequent 9 months.

Cytological investigation of longitudinal sections of the scales and basal plate of the mother bulb revealed meristematic cells at the bases of the scales during most stages of ontogenesis. Both the number of meristematic cells in these zones and their rate of cell division increased during conversion of the apical meristem (of the primary daughter bulb) to flowering in June, but they disappeared

during formation of flower primordia (July–August). They then reappeared at about the time when active growth of new leaves, roots, and flower began.

These zones of meristematic cells between scales are of considerable interest, since they may give rise to adventitious bulblets that form during normal bulb growth or that arise following induction treatments during commercial propagation. The appearance and disappearance of these zones during ontogenesis of *S. peruviana* is probably controlled by endogenous growth regulators, which have a profound influence on bulb growth and development (Saniewski and Kawa-Miszczak, 1992). Elevated levels of gibberellins during flower differentiation may inhibit adventitious bulblet initiation in *Hyacinthus orientalis* L. (Saniewski et al., 1978).

The correlation of bulb characteristics with developmental stage may be used to indicate the ontogenetic progress of the bulb. This facilitates the design of handling regimes that permit control of growth and development by manipulation of external factors, such as temperature, light, growth regulators, and retardants. In cooperation with a commercial grower, we have applied our observations on the growth and development of *S. peruviana* to develop a successful forcing program that extends flowering from mid-February through April.

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